# Vitamin D

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CHAPTER 89

# Vitamin D: Cancer and Differentiation

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## I. INTRODUCTION

The seco-steroid hormone 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] is the most potent natural metabolite of vitamin $D_3$ and is an important regulator of calcium homeostasis and bone metabolism via actions in intestine, bone, kidney, and parathyroid glands. 1,25-$(OH)_2D_3$ exerts its effects via an intracellular receptor that is a member of the steroid hormone receptor family (see Chapters 11–20 and 22 in this book). Throughout the last decades, it has become evident that the vitamin D receptor (VDR) is not limited to cells and tissues involved in regulation of calcium and bone metabolism but is also present in a wide variety of other cells and tissues including cancer cells of various origins. This led to a vast series of studies on the role of vitamin D in tumor cell growth regulation, treatment of cancer, and development of potent synthetic vitamin D analogs. Various specialized chapters will discuss in detail the effect of vitamin D on specific cancers (Chapters 89–97) and development and actions of vitamin D analogs (Chapters 80–88). In this chapter we aim to give an overview of the history and current stage and developments on vitamin D and cancer, regulation of tumor cells, possible mechanisms, and clinical applications.

## II. VITAMIN D AND CANCER

### A. Vitamin D Receptor

As exemplified in Table I, the VDR has also been demonstrated in a broad range of tumors and malignant cell types. For colon and breast cancer cells, an inverse relationship between VDR level and degree of differentiation has been described by some investigators [1,2]. VDR level is increased in ovarian carcinoma compared to normal ovarian tissue [3]. For colorectal cancer it was shown that VDR expression is associated with a more favorable prognosis in colorectal cancer [4]. A VDR immunoreactivity score showed an increase in breast carcinoma specimens compared to normal breast tissue but no clear relation with proliferative status could be assessed [5]. A later study by the same group showed that VDR expression is not a prognostic factor for breast cancer, but the strong VDR immunoreactivity in the breast cancer specimens supports the evidence for it to be a target for intervention [6]. Also in other studies no associations between VDR and clinical and biochemical parameters of breast cancer were found [7–12].

Albeit that the association studies on VDR expression and predictive and/or prognostic characteristics for cancer are so far not conclusive, the widespread distribution of the VDR in malignant cells indicates that regulation of cancer cell function might be a new target in the action of 1,25-$(OH)_2D_3$ and provides a biological basis for the epidemiological observations discussed in the next paragraph.

A recent observation put the VDR in relation to cancer in a whole new perspective. It was shown that VDR can function as a receptor for the secondary bile acid lithocholic acid. This compound is hepatotoxic and a potential enteric carcinogenic. Interestingly, both binding of lithocholic acid and vitamin D to the VDR results in induction of CYP3A, the enzyme that detoxifies lithocholic acid in the liver and intestine [13,14]; (see also Chapter 53). It is postulated that vitamin D and lithocholic acid, by binding to the VDR, activate a feed-forward catabolic pathway that increases CYP3A expression leading to detoxification of carcinogenic bile acids. A relation between the presence of VDR and carcinogenesis was recently also shown for the skin. Absence of VDR increased the sensitivity for chemically induced tumorigenesis [15].

### B. Epidemiology

In 1980 an epidemiological study based on indirect evidence suggested a relationship between vitamin D and cancer. This was derived from analyses of death

TABLE I VDR in Tumors and Malignant Cell Types

| | |
|---|---|
| Basal cell carcinoma | Myeloid leukemia |
| Breast carcinoma | Myeloma |
| Bladder cancer | Osteogenic sarcoma |
| Cervical carcinoma | Ovarian carcinoma |
| Colonic adenocarcinoma | Neuroblastoma |
| Colorectal carcinoma | Non-Hodgkin's lymphoma |
| Gall bladder carcinoma | Pancreatic carcinoma |
| Glioma cells | Parathyroid adenoma |
| Kaposi sarcoma | Pituitary adenoma |
| Lung carcinoma | Prostate carcinoma |
| Lymphocytic leukemia | Renal cell carcinoma |
| Malignant B-cell progenitors | Squamous cell carcinoma |
| Malignant melanoma | Transitional cell bladder carcinoma |
| Medullary thyroid carcinoma | Uterine carcinosarcoma |

rates from colon cancer, which tended to increase with increasing latitude and decreasing sunlight [16]. Later more direct evidence about a relation between vitamin D and colon cancer came from the inverse relationship between levels of serum 25-hydroxyvitamin $D_3$ [a 1,25-$(OH)_2D_3$ precursor] and incidence of colonic cancer [17,18]. In addition, a similar relationship between sunlight exposure, vitamin D, and the risk for fatal breast and prostate cancer has been suggested [19–23] (see Chapter 90). The relationship between sunlight exposure and cancer, especially with respect to vitamin D, has been carefully reviewed by Studzinski and Moore [24]. The dual relationship between sunlight and cancer is of interest and remains the subject of continuing studies [25–27]. A relationship between skin type and prostate cancer has been described [28–30] and recently an article on the skin, sunlight, vitamin D, and cancer has been presented from an evolutionary perspective [31].

The relationship between cancer, diet, and calcium intake and vitamin D has been addressed in several studies [32–37] (see Chapter 91). A Canadian study noted similar vitamin D intakes in breast cancer patients and control subjects [38]. Moreover, in a mouse model, no relationship was found between dietary intake of a wide range of doses of calcium or vitamin D and carcinogen-induced skin tumors [39]. A large Finish epidemiological study showed an association of low serum 25-hydroxy-vitamin $D_3$ with prostate cancer [40,41]. A study on intake of micronutrients suggested that vitamin D and calcium might interact with antioxidants like vitamin C and E in reducing colorectal cancer risk [42]. It is clear that sunlight exposure, vitamin D intake, and other dietary components such as calcium and fat should be considered as possibly interacting with one another when the relationship between vitamin D and cancer risk is assessed. The data on VDR as bile acid sensor and its postulated role in detoxification provide a direct biological basis for the relation between increased colon cancer and high-fat diets [43] and that colon cancer occurs in areas with higher prevalence of rickets [36]. In addition, mice lacking VDR have been reported to have a higher proliferation rate in the colon [44,45]. A survey of mutations in the VDR in osteosarcomas, several other sarcomas, nonsmall cell lung cancers, and a large number of cell lines representing many tumor types did not show that mutations or rearrangements in the VDR gene play a role in these cancers [46]. Aspects on sunlight and the epidemiology of vitamin D and calcium will be further discussed in greater detail in Chapters 90 and 91, respectively.

In the VDR gene several polymorphisms have been identified and studied in relation to various endpoints (discussed in Chapter 68). Throughout the last years, an increasing number of studies have studied the association of polymorphisms in the VDR and cancer. The first study showed an association between polymorphisms at the 3′ end of the VDR gene and prostate cancer [47]. This was shortly followed by a study showing an association of prostate cancer with variations in the 3′ poly-A stretch in the VDR gene [48]. Interestingly, the Odds Ratio for the VDR polymorphism was about twofold that of the one for the CAG repeat in the androgen receptor. This was followed by several others studies also showing associations of polymorphisms in the 3′ region of the VDR gene and prostate cancer, [49–55] albeit other studies couldn't confirm this [56–60]. For breast cancer both presence [61–66] and absence of association [67] with polymorphisms in the VDR gene have been reported. Also for colon cancer both presence [68,69] and absence [70] of an association with VDR polymorphisms have been reported. No association was reported with basal cell carcinoma [71]. A single study reported an association with the aggressive renal cell carcinoma [72], malignant melanoma [73], and another study on rectal cancer reported a correlation between VDR gene polymorphisms and erbB-2/HER-2 expression [74]. It should be concluded that so far the studies on VDR gene polymorphisms and cancer are far from conclusive. A major reason might be the limited size of most of the studies. More association studies on VDR gene polymorphisms and specific cancers are needed, which should be followed by a meta-analysis to definitively assess whether there is an association and if so, what is the size of the effect. Also, for studies on VDR gene polymorphisms, it is important to take into account the

impact of environmental factors. Diet, vitamin D intake, and sun exposure may modify the association of polymorphism and cancer risk. Interaction between vitamin D and calcium intake and cancer was also found in some of the VDR gene polymorphism studies [68,75–77]. Some studies reported decreased risk of prostate cancer [75] and colorectal adenomas [76] in those subjects with lower vitamin D levels and a particular VDR gene polymorphism. However, results of these studies are unusual in light of the fact that higher calcium and vitamin D intake are generally associated with a modestly reduced risk of colorectal neoplasia. Finally, most importantly it should be realized that except for the FokI translational start site polymorphism, all polymorphisms analyzed so far are anonymous, and functionality or linkage with functional polymorphisms should be proven. The 3′ polymorphisms have been shown to be in linkage with 3′-UTR polymorphisms, but no relation with VDR mRNA stability could be proven [78]. Detailed discussion of possible functional consequences of VDR gene polymorphisms and impact of vitamin D levels is beyond the scope of this chapter but will be addressed in Chapter 67.

## C. Growth and Development

In addition to the epidemiological studies and demonstration of vitamin D receptor in tumor cells, since the early 1980s there has also been an increasing amount of cell biological data supporting a role for vitamin D in cancer. Multiple studies have shown that at high concentrations ($10^{-9}$–$10^{-7}$ M) 1,25-$(OH)_2D_3$ inhibits the growth of tumor cells *in vitro*. It was demonstrated as early as 1981 that 1,25-$(OH)_2D_3$ inhibits the growth of malignant melanoma cells and stimulates the differentiation of immature mouse myeloid leukemia cells in culture [79–81]. 1,25-$(OH)_2D_3$ also induces differentiation of normal bone marrow cells (see Chapter 96). Immature bone marrow cells of the monocyte-macrophage lineage are believed to be the precursors of osteoclasts, and 1,25-$(OH)_2D_3$ induces differentiation of immature myeloid cells toward monocytes-macrophages and also stimulates the activation and fusion of some macrophages (discussed in Chapter 38). From these results, it has been postulated that 1,25-$(OH)_2D_3$ stimulates differentiation and fusion of osteoclast progenitors into osteoclasts [82–84]. Also, in the intestine, 1,25-$(OH)_2D_3$ has important effects on cellular proliferation and differentiation [85]. Thus, via stimulation of the differentiation inducing capacity of bone and interstitial cells, 1,25-$(OH)_2D_3$ may play an important role in the regulation of calcium and bone metabolism. These *in vitro* findings were followed by the *in vivo* observation that 1,25-$(OH)_2D_3$ prolongs the survival time of mice inoculated with myeloid leukemia cells [86]. As shown in Table II, over the years 1,25-$(OH)_2D_3$ has been shown to have beneficial effects in several other *in vivo* animal models of various types of cancers [87–109].

An important aspect and limitation of the treatment of cancer with 1,25-$(OH)_2D_3$ was revealed by this limited set of clinical trials (see section II.D); to achieve growth inhibition, high doses are needed (confirming the *in vitro* data), which can cause the side effect of hypercalcemia. This has prompted the development of analogs of 1,25-$(OH)_2D_3$ in order to dissociate the antiproliferative effect from the calcemic and bone metabolism effects (see Chapters 80–88) [110,111]. Although the precise mechanism is not completely understood, at the moment several 1,25-$(OH)_2D_3$ analogs are available that seem to fulfill these criteria. In Table III the *in vivo* animal studies using 1,25-$(OH)_2D_3$ analogs on various cancer types are summarized [97,103,104,106–109,112–129].

## D. Clinical Studies

Considering the calcemic actions of 1,25-$(OH)_2D_3$ up to this point in time only a few clinical trials of vitamin D compounds in cancer have been performed. Alfacalcidol (1α-hydroxyvitamin $D_3$; 1α-$(OH)D_3$), which is converted to 1,25-$(OH)_2D_3$ *in vivo*, caused a beneficial response in low-grade non-Hodgkin's lymphoma patients [130,131]. Also, with alfacalcidol, transient improvement in peripheral blood counts was seen in patients with myelodysplasia; however, half of the patients developed hypercalcemia [132]. Another study reported a sustained hematological response in six myelodysplasia patients treated with high doses of alfacalcidol [133]. These patients were restricted in their dietary calcium intake; nevertheless, four patients developed hypercalcemia due to increased bone resorption. With respect to treatment of cutaneous T-cell lymphoma with a combination of 1,25-$(OH)_2D_3$ and retinoids, contrasting results have been obtained. It has been suggested that the variability was due to differences in phenotype of the various lymphomas [134–138]. A study on early recurrent prostate cancer showed that daily treatment with 1,25-$(OH)_2D_3$ slowed the rise in prostate-specific antigen, but treatment coincided with hypercalcemic affects [139]. Using a regime of weekly treatment with high-dose calcitriol was found to be safe, but didn't result in a significant reduction in prostate-specific antigen (PSA) in prostate cancer cells [140]. Two studies were specifically designed to examine the route of application and calcemic response in patients with advanced malignancies [141,142].

TABLE II *In Vivo* Effects of 1,25-$(OH)_2D_3$ and 1α-$(OH)D_3$ in Animal Models of Cancer[a]

| Tumor | Model | Effect | Refs. |
|---|---|---|---|
| Adenocarcinoma | CAC-8 cells injected in nude mice | Reduction in tumor volume | [107] |
| Breast | NMU- and DMBA-induced breast cancer in rats | Tumor suppression | [93,96] |
| Colon | Human colon cell line implanted into nude mice; DMH-induced colon cancer in rats; APCmin mice | Tumor suppression; reduction of the incidence of colon adenocarcinomas; decrease in polyp number and tumor load | [90,92,95,371] |
| Kaposi sarcoma | KS Y-1 cells implanted in nude mice | Tumor growth retardation | [105] |
| Leydig tumor | Leydig cell tumor implanted into rats | Tumor suppression | [97] |
| Lung | Implantation of lewis lung carcinoma into mice | Reduction of the number of metastases (without suppression of primary tumor); tumor suppression; increased antitumor immunity | [87,99,101,102] |
| Melanoma | Human melanoma cells implanted into nude mice | Tumor suppression | [90] |
| Osteosarcoma | Human osteosarcoma cells implanted into nude mice | Tumor suppression | [98] |
| Prostate | Dunning MAT LyLu rat prostate model; LNCaP xenografts in nude mice; PAIII tumors in Lobund-Wistar rats | Reduction in lung metastasis; tumor suppression | [103,104,106,108,109] |
| Retinoblastoma | Retinoblastoma cell line implanted into nude mice; transgenic mice with retinoblastoma | Tumor suppression | [91,94] |
| Walker carcinoma | Walker carcinoma cells injected in rats | Tumor suppression | [100] |
| Skin | DMBA/TPA-induced skin tumors in mice | Inhibition of tumor formation | [88,89] |

[a]The dosage, duration of treatment, diet, and effects on serum/urinary calcium vary among the studies. NMU, Nitrosomethylurea; DMBA, 7,12-dimethylbenz[a]anthracene; DMH, 1,2-dimethylhydrazine dihydrochloride; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Clinical trials using vitamin D analogs have been initiated over the last years. However, these were mostly limited clinical trials focusing on small groups of patients for whom regular treatment has failed. Only data from a few studies has been published. The analog calcipotriol (MC903) has been used for topical treatment of advanced breast cancer; however, several of the patients still developed hypercalcemia [143]. More recent studies have been published on advanced breast cancer [144] and pancreatic cancer [145] but the clinical results were limited. In a single case of Kaposi sarcoma and topical application of calcipotriol (Daivonex/Dovonex/MC903), good success in tumor regression was reported [105]. In Chapter 97 the current clinical status of 1,25-$(OH)_2D_3$ and its analogs as therapeutic agents for cancer will be discussed in greater detail.

## E. Angiogenesis and Metastasis

For the tumor suppressive activity of vitamin $D_3$ compounds *in vivo,* besides growth inhibition, two additional actions may be involved. First, angiogenesis is an essential requirement for the growth of solid tumors. Compounds that inhibit angiogenesis might therefore contribute to antitumor therapy. Antiangiogenic drugs may cause inhibition of tumor progression, stabilization of tumor growth, tumor regression, and prevention of metastasis. Antiangiogenic effects may play a role in the tumor suppressive activity of vitamin $D_3$ compounds. Two studies reported an antiangiogenic effect of 1,25-$(OH)_2D_3$ and the analog 22-oxacalcitriol using different experimental model systems [115,146]. In addition, it was shown that 1,25-$(OH)_2D_3$ inhibits angiogenesis induced by the human papilloma virus type 16 (HPV16)- or HPV18-containing cell lines HeLa, Skv-e2, and Skv-el2 when intradermally injected into immunosuppressed mice [147]. Also, with the non-virus-transformed human cell lines T47-D (breast carcinoma) and A431 (vulva carcinoma), similar results were obtained [148]. In these studies the mice were treated for 5 days with 1,25-$(OH)_2D_3$ prior to the injection of tumor cells. The effect of 1,25-$(OH)_2D_3$ on angiogenesis may be due to inhibition of tumor cell proliferation, resulting in fewer angiogenic cells.

TABLE III *In Vivo* Effects 1,25-$(OH)_2D_3$ Analogs in Animal Models for Cancer[a]

| Analog | Model | Antitumor effect | Refs. |
|---|---|---|---|
| 1,25-$(OH)D_2$ | Retinoblastoma | Tumor suppression | [128] |
| 1,25-$(OH)D_5$ | Breast | Tumor suppression | [129] |
| CB966 | Breast | Tumor suppression | [114] |
| CB1093 | Prostate | Tumor suppression | [108] |
| | | No effect on angiogenesis | |
| DD-003 | Colon | Tumor suppression | [120] |
| EB1089 | Adenocarcinoma | Tumor suppression | [107] |
| EB1089 | Breast | Tumor suppression | [114,116,125,316] |
| EB1089 | Colon | Tumor suppression | [124] |
| EB1089 | Hepatocellular carcinoma | Inhibition of tumor incidence | [372] |
| EB1089 | Leydig cell tumor | Tumor suppression | [97] |
| EB1089 | Prostate | Tumor suppression | |
| | | Reduction lung metastases | [104,106,108,109,126,127] |
| | | No effect on angiogenesis | |
| KH1060 | Prostate | Tumor suppression | [109] |
| LG190119 | Prostate | Tumor suppression | [106] |
| OCT | Breast | Tumor suppression | [113,118] |
| OCT | Breast | Tumor suppression | [115] |
| OCT | Breast | Tumor suppression | [118] |
| OCT | Colon | Decreased tumor incidence | [121] |
| MC903 | Breast | Tumor suppression | [117] |
| Ro 23-7553 | Prostate | Tumor suppression | [122] |
| Ro 23-7553 | Leukemia | Increased survival | [112] |
| Ro 24-5531 | Breast | Decreased tumor incidence | [119] |
| Ro 24-5531 | Colon | Decreased tumor incidence | [123] |
| Ro-25-6760 | Prostate | Tumor suppression | [103] |
| Ro-26-9114 | Colon | Decrease in polyp number and tumor load | [371] |
| Ro-26-9114 | Prostate | Tumor suppression | [109] |

[a]MC903, 1,24-dihydroxy-22-ene-24-cyclopropyl-vitamin $D_3$; CB966, 24a,26a,27a-tri-homo-1α,25-dihydroxyvitamin $D_3$; CB1093, 20-epi-22(S)-ethoxy-23yne-24a, 26a,27a-trihomo-1α,25-dihydroxyvitamin $D_3$; DD-003,22(S)-24-homo-26,26,26,27,27,27-hexafluoro-1α,22,25-trihydroxyvitamin $D_3$; EB1089, 22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin $D_3$; OCT, 22-Oxacalcitriol; Ro 23-7553, 1,25-dihydroxy-16-ene-23-yne-vitamin $D_3$; Ro 24-5531, 1,25-dihydroxy-16-ene-23-yne-26,27-hexafluorovitamin $D_3$. Ro 26-9114, 1α,25-$(OH)_2$-16-ene-19-nor-24-oxo-$D_3$.

However, inhibition of angiogenesis could also be observed when the tumor cells were treated *in vitro* with 1,25-$(OH)_2D_3$ and, after cell washing, were injected into mice [148]. Under these conditions both control and 1,25-$(OH)_2D_3$-treated mice were injected with similar numbers of cells. Therefore, these data indicate that 1,25-$(OH)_2D_3$ inhibits the release of angiogenic factors (vascular endothelium growth factor, transforming growth factor-α, basic fibroblast growth factor, epidermal growth factor, etc.) or stimulates antiangiogenic factors. 1,25-$(OH)_2D_3$ treatment caused a reduction in the angiogenic signaling molecule, angiopoietin-2 in squamous cell carcinoma and radiation-induced fibrosarcoma-1 cells [149]. In retinoblastomas in mice, 1,25-$(OH)_2D_3$ has also been shown to reduce angiogenesis [150]. A recent study by Oades *et al.*, however, showed that the 1,25-$(OH)_2D_3$ analogs EB1089 and CB1093 inhibited tumor growth in two prostate animal models but did not inhibit angiogenesis in a rat aorta assay [108]. Whether this indicates that vitamin D affects angiogenesis in a tumor situation and not in a nonmalignant condition is not clear. This may resemble the effects of endostatin, which inhibits pathological but not normal

vascularization [151,152]. In support of this is the finding that 1,25-$(OH)_2D_3$ and its analogs EB1089, Ro-25-6760, and ILX23-7553 potently inhibit growth of endothelial cells derived from tumors, but are less potent against normal aortic or yolk sac endothelial cells [149]. Finally, an interesting observation is deglycosylated vitamin D–binding protein (DBP-maf) has also been reported to inhibit angiogenesis [153,154] and to inhibit growth of pancreatic tumor in nude mice [154]. Whether 1,25-$(OH)_2D_3$ may interfere with DBP-maf in tumor growth inhibition and antiangiogenesis remains to be established. Interaction with another factor, interleukin-12, in the inhibition of angiogenesis has been reported [155].

The second mechanism of antitumor activity, which may be related to angiogenesis, is metastasis. Metastasis is the primary cause of the fatal outcome of cancer diseases. A study by Mork Hansen *et al.* indicated that 1,25-$(OH)_2D_3$ may be effective in reducing the invasiveness of breast cancer cells [156]. They showed that 1,25-$(OH)_2D_3$ inhibited the invasion and migration of a metastatic human breast cancer cell line (MDA-MB-231) using the Boyden chamber invasion assay. In support of this, it was shown that 1,25-$(OH)_2D_3$, KH1060, EB1089, and CB1093 inhibited secretion of tissue-type and urokinase plasminogen activator and increased plasminogen activator inhibitor 1 in the MDA-MB-231 metastatic breast cancer cells [157]. In an *in vivo* study, it was shown that 1,25-$(OH)_2D_3$ reduces metastasis to the lung of subcutaneously implanted Lewis lung carcinoma cells [101]. In two animal models of prostate cancer, 1,25-$(OH)_2D_3$ and the analogs EB1089 and RO25-6760 inhibited lung metastases [103,104]. In these models, the tumors were implanted subcutaneously and therefore, in contrast to the model of direct tumor cell injection in the left ventricle [158], no bone metastases occurred. However, a fact to be considered in relation to metastasis is that bone is the most frequent site of metastasis of advanced breast and prostate cancer. There are some indications from clinical studies that bone metastases develop preferentially in areas with high bone turnover [159,160]. In contrast, agents that inhibit bone resorption have been reported to reduce the incidence of skeletal metastasis [161]. As 1,25-$(OH)_2D_3$ may stimulate bone turnover, treatment of cancer with 1,25-$(OH)_2D_3$ might theoretically increase the risk of skeletal metastases. This aspect of 1,25-$(OH)_2D_3$ therapy certainly needs further study. In this aspect, the use of vitamin $D_3$ analogs with reduced calcemic activity or treatment with vitamin $D_3$ in combination with other compounds to reduce bone turnover (see Section IV) may be helpful. The data obtained so far on angiogenesis and metastasis indicate that these two processes are part of the spectrum of mechanisms by which vitamin $D_3$ exerts its anticancer activity.

## F. Parathyroid Hormone-Related Peptide

1,25-$(OH)_2D_3$ and parathyroid hormone (PTH) mutually regulate synthesis and secretion of one another. Production and secretion of PTH are inhibited by 1,25-$(OH)_2D_3$ via a transcriptional effect, and a vitamin D responsive element (VDRE) in the promoter of the PTH gene has been identified [162,163] (see Chapter 30). Parathyroid hormone-related peptide (PTHrP) was initially isolated from several carcinomas and is responsible for the humoral hypercalcemia of malignancy syndrome [164]. Although originally identified in carcinomas, PTHrP has also been identified in normal cells (see Chapter 43).

In normal human mammary epithelial cells, 1,25-$(OH)_2D_3$ did not affect basal but inhibited growth factor-stimulated PTHrP expression via an effect on transcription [165]. In normal keratinocytes 1,25-$(OH)_2D_3$ had no effect on PTHrP secretion in basal culture conditions [166], but did inhibit growth factor-stimulated PTHrP production as well [167]. Likewise, 1,25-$(OH)_2D_3$, as well as the analogs 22-oxacalcitriol and MC903, inhibited PTHrP secretion in immortalized human keratinocytes (HPK1A), but this inhibition was less in the more malignant ras-transfected clone HPK1A-ras [168,169]. 1,25-$(OH)_2D_3$ and the analogs EB1089 and 22-oxacalcitriol inhibit the PTHrP gene transcription in and release from the squamous cancer cell line NCI H520 [170]. In addition, in the human T-cell lymphotrophic virus type I (HTLV-I)-transfected T-cell line MT-2, 1,25-$(OH)_2D_3$ and 22-oxacalcitriol did inhibit PTHrP gene expression and PTHrP secretion [171]. In rat H-500 Leydig tumor cells [172], and PC-3 prostate cancer cells 1,25-$(OH)_2D_3$ inhibited PTHrP secretion. It was suggested that this might play a role in the growth inhibition by vitamin D as PTHrP stimulates prostate cancer growth, tumor invasion, and metastasis [173–175]. *In vivo* observations comparable to these *in vitro* observations have also been made. When these H-500 Leydig tumor cells were implanted in Fisher rats, treatment with 1,25-$(OH)_2D_3$ and the analog EB1089 resulted in reduced levels of tumor PTHrP mRNA and PTHrP serum levels [97]. EB1089 also reduced serum levels of PTHrP in nude mice implanted with squamous cancer cells [176]. In Fisher rats implanted with the Walker carcinoma, 1,25-$(OH)_2D_3$ caused a decrease in serum PTHrP, but the ratio of PTHrP levels and tumor weight was similar in rats receiving vehicle or 1,25-$(OH)_2D_3$. The data point to an indirect effect on PTHrP via growth inhibition. However, the PTHrP mRNA levels appeared to be decreased by 1,25-$(OH)_2D_3$ [100]. In nude mice bearing the FA-6 cell line of a pancreas carcinoma lymph node metastasis, 22-oxacalcitriol inhibits PTHrP gene expression, which is related to inhibition of

tumor-induced hypercalcemia [177]. Together, the overall picture that emerges from these studies is that an important additional anticancer effect of vitamin $D_3$ and analogs could be the inhibition of the humoral hypercalcemia of malignancy.

In contrast to these inhibitory effects in human tumor cells and tumor models, a stimulatory effect of 1,25-$(OH)_2D_3$ and EB1089 on PTHrP gene transcription and PTHrP production by a canine oral squamous carcinoma cell line (Sec 2/88) has been observed [178,179]. Also in an *in vivo* model of canine adenocarcinoma CAC-8 implanted in nude mice, stimulation of PTHrP by 1,25-$(OH)_2D_3$ and EB1089 was observed [179]. These data indicate that the effect of vitamin D and analogs on canine tumors differs from that on human tumors.

## III. VITAMIN D EFFECTS ON TUMOR CELLS

### A. Cell Cycle

It has now been well established that vitamin D inhibits growth of cells by interfering with the cell cycle. Proliferating cells progress through the cell cycle, which comprises the $G_0/G_1$ phase (most differentiated, nondividing cells are in the $G_1$ phase), the S phase in which new DNA is synthesized, and the $G_2$ phase, which is followed by mitosis (M phase) whereon the cells reenter the $G_0/G_1$ phase. In most of the cells studied so far, treatment with 1,25-$(OH)_2D_3$ and its analogs results in a blockade at a specific checkpoint, i.e., the restriction point (R), in the $G_1$ phase limiting the transition of $G_1$ to S and reducing the number of cells in S phase. Some studies also have examined the effect on the $G_2$ phase, but these results are somewhat more diverse. In general it can be concluded that blocking the transition from the $G_0/G_1$ phase to the S phase plays an important role in the growth inhibitory effect of 1,25-$(OH)_2D_3$.

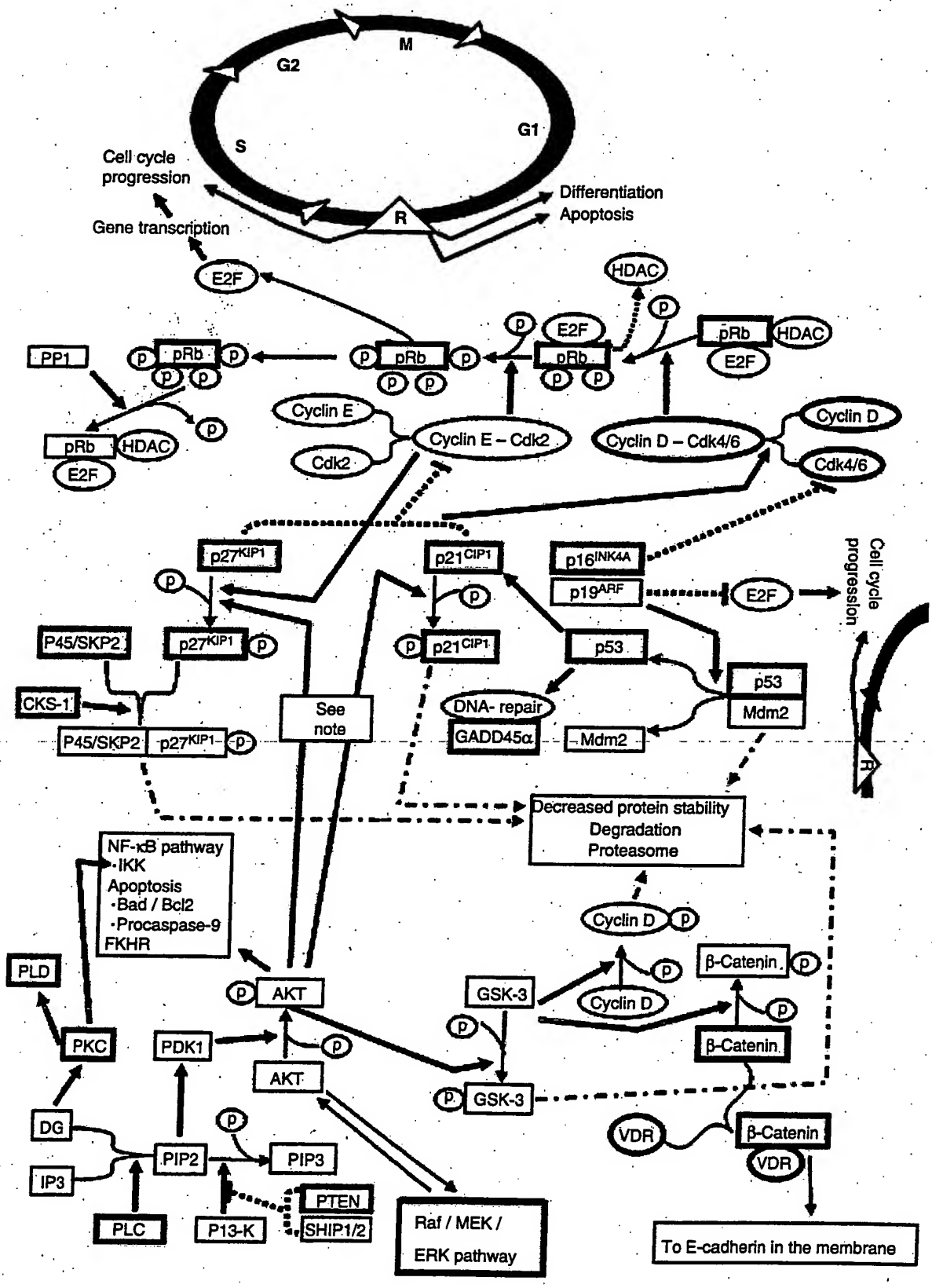
In the regulation of the cell cycle, numerous genes and proteins have been described. It is beyond the scope of this chapter to discuss in detail the regulation of all of the genes/proteins by vitamin D. In Fig. 1, an overview is given of the interacting genes/proteins that are involved in intracellular signaling and regulating the cell cycle. These genes and proteins are part of the cascade of events on which vitamin D exerts its effects. The components shown to be regulated by vitamin D are indicated. Figure 1 is a compilation of data present so far; it is important to realize that probably not all genes/proteins are affected by vitamin D in all tumor cells. However, in this way one gets an overview of the broad range of effects of vitamin D on intracellular signaling pathways involved in regulation of (tumor) cell growth. More details on the regulation of the cell cycle will be discussed in several other chapters, especially Chapter 92.

Besides its effects on cell cycle regulation, vitamin D has recently been implicated to be involved in control of genomic stability [180]. 1,25-$(OH)_2D_3$ has been reported to inhibit hepatic chromosomal aberrations and DNA strand breaks [181]. This is supported by the finding that 1,25-$(OH)_2D_3$ and EB1089 stimulated the expression of GADD45, which stimulates DNA repair [182] and might be coupled to release of p53 from Mdm2 (see Fig. 1).

#### 1. (ONCO)GENES AND TUMOR SUPPRESSOR GENES

Oncogenes and tumor suppressor genes generally are involved in control of the cell cycle and apoptosis (see Chapter 92). One of the most widely studied oncogenes in relation to vitamin D is *c-myc*. C-Myc suppresses expression of cell cycle/growth arrest genes gas1, p15, p21, p27, and gadd34, -45, and -153 [183]. C-Myc has been postulated to play an early role in the following cascade of events in $G_1$: cyclins activate cyclin-dependent kinases (CDKs), which in turn can phosphorylate the retinoblastoma tumor suppressor gene product ($p110^{RB}$), resulting in transition from $G_1$ to S phase (see Fig. 1). In HL-60 cells, breast cancer cells, and several other cell types, 1,25-$(OH)_2D_3$ has been reported to decrease *c-myc* oncogene expression [184–189]. Analysis of HL-60 sublines showed a relation between reduction of c-*myc* expression and inhibition of proliferation [190]. Similar observations were made for neuroblastoma cells treated with 1,25-$(OH)_2D_3$, EB1089, and KH10560 [191]. We did not observe a 1,25-$(OH)_2D_3$-induced change in *c-myc* expression in MCF-7 and ZR-75.1 breast cancer cells while they were both growth inhibited [192], and a similar observation has been made for the colon-adenocarcinoma CaCo-2 cell line [193].

Nontransformed embryonic fibroblasts are growth inhibited by 1,25-$(OH)_2D_3$, whereas *c-myc* is not changed or is even increased [194,195]. In the MG-63 osteosarcoma cell line, 1,25-$(OH)_2D_3$ has been shown to enhance *c-myc* expression [196], whereas we observed growth inhibition by 1,25-$(OH)_2D_3$ [197]. These data show that regulation of c-myc expression may be part of growth inhibition by vitamin D, but that this is not generally applicable to all cells. 1,25-$(OH)_2D_3$ has also been reported to regulate expression of other oncogenes, like *c-myb*, *c-fos*, *c-fms*, *c-fra1*, *c-jun*, *junD*, *c-Ki-ras*, *N-ras*, *c-src* [189,198–203]; however, these data are rather limited. Nevertheless, it is clear that 1,25-$(OH)_2D_3$ has effects on the expression of various oncogenes. The data so far are not conclusive with respect to which genes are crucial in the growth inhibitory action of 1,25-$(OH)_2D_3$. This can be attributed to the fact that these (proto)oncogenes encode for transcription factors,


M
G2
G1
S
R
Cell cycle progression
Gene transcription
Differentiation
Apoptosis
E2F
HDAC
PP1
pRb
p
Cyclin E
Cdk2
Cyclin E – Cdk2
Cyclin D – Cdk4/6
Cyclin D
Cdk4/6
p27KIP1
p21CIP1
p16INK4A
p19ARF
P45/SKP2
CKS-1
See note
p53
Mdm2
DNA- repair
GADD45α
Cell cycle progression
Decreased protein stability
Degradation
Proteasome
NF-κB pathway
·IKK
Apoptosis
·Bad / Bcl2
·Procaspase-9
FKHR
PLD
PKC
PDK1
AKT
GSK-3
β-Catenin
DG
IP3
PIP2
PIP3
PTEN
SHIP1/2
PLC
P13-K
Raf / MEK /
ERK pathway
VDR
To E-cadherin in the membrane

growth factor receptors, or components or intracellular signaling cascades. The effects of these may differ between cells dependent on presence or absence of additional cell type specific conditions. Therefore, their postulated role is often complex. For example, increased *c-myc* expression can be related to induction of apoptosis but also to stimulation of cell cycle progression.

In contrast to the oncogenes, the effect of 1,25-$(OH)_2D_3$ on the retinoblastoma tumor suppressor gene is much clearer. This may be related to the fact that, in contrast to oncogenes, retinoblastoma and p53 take well-defined positions in the control of cell cycle and DNA repair (see Fig. 1). The $p110^{RB}$ retinoblastoma gene product can either be phosphorylated or dephosphorylated. In the phosphorylated form, it can activate several transcription factors and cause transition to S phase and DNA synthesis. In human chronic myelogenous leukemia cells [204], breast cancer cells [205], and HL-60 cells [206,207], 1,25-$(OH)_2D_3$ caused a dephosphorylation of $p110^{RB}$, which is related to growth inhibition and cell cycle arrest in $G_0/G_1$ and also in $G_2$ [207]. In leukemic cells, 1,25-$(OH)_2D_3$ also caused a reduction in the cellular level of $p110^{RB}$ [204,206]. In nontransformed keratinocytes, 1,25-$(OH)_2D_3$ induced dephosphorylation of $p110^{RB}$ as well [208]. The other major tumor suppressor gene is p53. For leukemic U937 cells, it was reported that presence of p53 is important for 1,25-$(OH)_2D_3$-induced differentiation [209]. In rat glioma cells, 1,25-$(OH)_2D_3$ induces expression of p53 [210]. However, 1,25-$(OH)_2D_3$ can inhibit cell growth and induce differentiation in cancer cells with defective p53 [211] and also p53-independent induction of apoptosis by EB1089 has been demonstrated [212]. These latter observations might be explained by the fact that vitamin D also interferes at levels in the cascade of cell cycle control down-stream of p53 (see Fig. 1). Recently, an additional interesting relationship between tumor suppressor genes and vitamin D has recently been shown for the Wilms' tumor suppressor gene WT1. This zinc-finger containing transcription factor induces transcription of the VDR gene [213].

Several interesting additional genes and vitamin D targets in cancer treatment should be mentioned. First in 1994 Chen and DeLuca isolated and characterized a vitamin D–induced gene in HL-60 cells [214]. This protein, vitamin D-up-regulated protein (VDUP1), is a thioredoxin-binding protein-2 [215]. Thioredoxin has several roles in processes such as proliferation or apoptosis. It also promotes DNA binding of transcription factors such as NF-κB, AP-1, p53, and PEBP2. In addition, overexpression of thioredoxin suppresses the degradation of IκB and the transactivation of NF-κB, whereas overexpression of nuclear-targeted thioredoxin exhibits the enhancement of NF-κB-dependent transactivation [216]. However, it is only in more recent studies that a relationship between VDUP1 and cancer has been established. The expression of VDUP1 was found to correlate with malignant status of colorectal and gastric cancers [217]. 5-fluorouracil, which is widely used for treatment of colon cancer, induces VDUP1 expression in the SW620 colon cancer cell line [218]. In smooth muscle cells and cardiomyocytes VDUP1 inhibits proliferation and is involved in induction of apoptosis [219,220]. An association with vitamin D effects on cancer is made by two recent studies showing induction of VDUP1 by 1,25-$(OH)_2D_3$ in tumor cells and that VDUP1 induces cell cycle arrest [221,222]. Moreover, interaction with histone deacetylase (HDAC; see Fig. 1), promyelocytic leukemia zinc-finger (PLZF) was demonstrated. Interestingly and further complicating the story, PLZF inhibits 1,25-$(OH)_2D_3$ induced differentiation of U937 leukemic cells by binding to the VDR and inhibiting gene transcription [223,224]. Interestingly, the gene, DRH1, was cloned from hepatocellular carcinoma, and its expression was strongly reduced in cancer tissue compared to normal liver [225]. DRH1 has a 41% homology with VDUP1. Whether this points to a new family of cancer genes remains to be established, but it certainly opens new venues for intervening in cancer cell growth.

FIGURE 1 Schematic representation summarizing the intracellular pathways and signaling pathways involved regulation of the cell cycle shown to be regulated by 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs in regulating cell proliferation. Targets shown to be affected by 1,25-$(OH)_2D_3$ and/or its analogs are indicated in the bold boxes and ovals. Bold arrows and fine dotted lines indicate stimulation and inhibition, respectively. Coarse dotted lines indicate processing to the proteasome. p indicates phosphorylation. The effects on these cellular targets are not demonstrated in all types of cancer cells but this diagram is aimed to give an overview of demonstrated targets and potential targets. NOTE: Dependent on the site of phosphorylation proteins can either be destabilized or degraded or be stabilized and activated. For example: phosphorylation of p21 at T145 by AKT leads to degradation while phosphorylation of S146 by AKT leads to increased stability.
Abbreviation used: AKT (PKB), Protein kinase B; Bad, BCL2-antagonist of cell death; Bcl2, B-cell leukemia/lymphoma 2; Cdk, Cyclin-dependent kinase; CKS-1, Cyclin kinase subunit 1; DG, Diacylglycerol; E2F, Transcription factor; ERK, Extracellular-signal regulated kinase; FHKR (AFX/FOX), Forkhead family of transcription factors; GSK-3, Glycogen synthase kinase-3; HDAC, Histone deacetylase; IKK, I-κB kinase; IP3, Inositol 1,4,5-trisphosphate; Mdm2, Mouse double minute 2; MEK, Raf-1-MAPK/ERK kinase; PDK1, Phosphatidylinositol-dependent kinase 1; PI3-K, Phosphatidyl inositol 3 kinase; PIP2, Phosphatidylinositol (4,5)-phosphate; PIP3, Phosphatidylinositol (3,4,5) phosphate; PKC, Protein kinase C; PLC, Phospholipase C; PLD, Phospholipase D; PP!, Protein phosphatase 1-like protein; pRB, Retinoblastoma protein; PTEN, Phosphatase and tensin homologue; SHIP 1 and 2, Src homology 2 (SH2) containing phosphatases 1 and 2; SKP2, Ubiquitin ligase; VDR, Vitamin D receptor.

Second, an additional therapeutic target for vitamin D compounds might be regulation of enzymes involved in estrogen and androgen synthesis and metabolism [226–229]. Third, telomerase activity provides a mechanism for unlimited cell division. In HL-60 cells, 1,25-$(OH)_2D_3$ inhibits telomerase activity [230]. Fourth, the homeobox genes may prove to be a major target for vitamin D action in cancer, but this possibility remains to be elucidated. In a differential expression screen using the human U937 leukemic cells, the HoxA10 gene was shown to be regulated by 1,25-$(OH)_2D_3$ [231].

It is to be expected that as a result of the increasing application of large scale microarray gene expression analyses, a vast number of new cell cycle and vitamin D regulated genes will be identified and add to the unraveling and understanding of vitamin D control of cancer cell proliferation [232–235].

## B. Apoptosis

A block in the cell cycle preventing transition into S phase may cause cells to go either into apoptosis or to enter a specific differentiation pathway (see Chapter 93). What exactly determines the decision of apoptosis or differentiation remains to be elucidated. It is suggested that early $G_1$ phase may be the point at which switching between cell cycle progression and induction of apoptosis occurs [236,237].

Induction of apoptosis, an orderly and characteristic sequence of biochemical, molecular, and structural changes resulting in the death of the cell [238], is a mechanism by which 1,25-$(OH)_2D_3$ inhibits tumor cell growth and may contribute to tumor suppression and explain the reduction in tumor volume found in various *in vivo* animal studies (see Section II.C).

1,25-$(OH)_2D_3$ has been shown to regulate expression of apoptosis genes and to induce apoptosis of cancer cells of various origins. For example, 1,25-$(OH)_2D_3$ and the analog Ro 25-6760 cause a cell cycle block in HT-29 human colon cancer cells, resulting in growth inhibition and induction of apoptosis [239]. The *bcl-2* oncogene decreases the rate of programmed cell death [240,241]. However, protection of HL-60 cells against apoptosis occurred despite down-regulation of *bcl-2* gene expression [242]. In several breast cancer cell lines (MCF-7, BT-474, MDA-MB-231) 1,25-$(OH)_2D_3$ and the analogs KH1060 and EB1089 decreased *bcl-2* expression [211,243]. The analog CB1093 reduced *bcl-2* expression in MCF-7 cells associated with the induction of apoptosis [244]. However, only in MCF-7 cells has this change in *bcl-2* expression been accompanied by apoptosis. Effects on other genes/proteins have also been reported [245], and microarray gene expression analyses and differential screening will also definitively reveal additional vitamin D targets in regulating apoptosis [246].

A central role for apoptosis in the action of 1,25-$(OH)_2D_3$ is unclear because growth inhibition of several other breast cancer cells appeared to be independent of apoptosis [211]. Also, MCF-7 cells that showed growth inhibition by 1,25-$(OH)_2D_3$ could, after removal of the hormone, again be stimulated to grow, implying transient growth inhibition and not cell death [247]. Stable transfection of leukemic U937 cells with the wild-type p53 tumor suppressor gene resulted in a reduced growth rate and produced cells that can undergo either apoptosis or maturation. In these cells 1,25-$(OH)_2D_3$ protects against p53-induced apoptosis and enhances p53-induced maturation [209]. In two independent studies with HL-60 cells, 1,25-$(OH)_2D_3$ was found either to protect against or to have no effects on apoptosis [242,248]. Vitamin D protection against apoptosis was also detected in human U937 leukemic cells treated tumor necrosis factor α [249]. Absence of a vitamin D effect on apoptosis might be explained by the expression of the antiapoptotic protein BAG-1 p50 isoform. This protein has been shown to bind to the VDR and block vitamin D–induced transcription [250]. The presence of additional interacting factors might also be important for the eventual effect on apoptosis as in the study with HL-60 cells, which in the presence but not the absence of 9-cis-retinoic acid, 1,25-$(OH)_2D_3$ did induce apoptosis [248]. The role of vitamin D interaction with other factors will be discussed in more detail in Section IV. In summary, the data obtained so far show that 1,25-$(OH)_2D_3$-induced growth inhibition can be related to apoptosis in some cases, but that growth inhibition is frequently observed to be independent of apoptosis. Possibly in these latter cases, induction of differentiation is more prominent. The factor that decides whether cells undergo apoptosis or differentiation is unclear but is probably dependent on cell cycle stage, presence of other factors, and levels of expression of oncogenes and tumor suppressor genes. An interesting phenomenom to be studied concerning vitamin D and apoptosis is calbindin 28K. Calbindin 28K is a well-known vitamin D–induced protein that has recently been shown to inhibit apoptosis [251]. It is tempting to speculate that calbindin 28K plays a role in the decision whether vitamin D induces cells to differentiate or to go into apoptosis or that it is involved when 1,25-$(OH)_2D_3$ protects against apoptosis (see Chapter 42).

## C. Differentiation

In addition to proliferation and apoptosis, the third major cellular process is differentiation. As described

above for the classic actions of 1,25-$(OH)_2D_3$ related to calcium homeostasis, effects on cell differentiation and proliferation are involved. The coupling between proliferation and differentiation has been most widely studied for cells of the hematopoietic system (Chapter 96) and keratinocytes (Chapter 35). In general, 1,25-$(OH)_2D_3$ inhibits proliferation and induces differentiation along the monocyte-macrophage lineage. Rapidly proliferating and poorly differentiated keratinocytes can be induced to differentiate by 1,25-$(OH)_2D_3$. A further relationship between the vitamin $D_3$ system and differentiation is demonstrated by the fact that in poorly differentiated keratinocytes 1,25-$(OH)_2D_3$ production and vitamin D receptor levels are high, whereas after induction of differentiation these levels decrease [252], and in melanoma cells 1,25-$(OH)_2D_3$ stimulates melanin production [253]. Effects on differentiation have also been reported for other cell types. Inhibition of prostate cancer cell proliferation is paralleled by an increased production of prostate specific antigen [254–257]. In the BT-20 breast cancer cells 1,25-$(OH)_2D_3$ induced morphological changes indicative for differentiation [258]. In several breast cancer cell lines, the stimulation of differentiation has been established by determining lipid production by the cells [211]. In this study, Elstner *et al.* demonstrated an uncoupling between effects on proliferation and differentiation. In two breast cancer cell lines, 1,25-$(OH)_2D_3$ and various analogs induced differentiation even though the cells were resistant to cell cycle and antiproliferative effects. This finding, together with data obtained with human myelogenous leukemia cells, [204] suggests a dissociation between the cellular vitamin $D_3$ pathways involved in regulation of differentiation and proliferation (see also Section V). For a HL-60 subclone, a similar observation was made [190], and in another HL-60 subclone the induction of differentiation was found to precede the $G_0/G_1$ cell cycle block. In contrast to the above-mentioned observations on stimulation of differentiation, 1,25-$(OH)_2D_3$ inhibits erythroid differentiation of the erythroleukemia cell line K562 [186], and 1,25-$(OH)_2D_3$ inhibits Activin A-induced differentiation of murine erythroleukemic F5-5 cells [259]. Although precise relationships among growth inhibition, cell cycle effects, and apoptosis are unclear, it can be concluded that an important effect of vitamin $D_3$ on both normal and malignant cells is induction of differentiation.

## D. Growth Factors and Growth Factor Receptors

Besides regulation of cell cycle-related oncogenes and tumor suppressor genes, interaction with tumor- or stroma-derived growth factors is important for growth inhibition. Stimulation of breast cancer cell proliferation by coculture with fibroblasts is inhibited by 1,25-$(OH)_2D_3$ [260]. A good candidate to interact with the 1,25-$(OH)_2D_3$ action is transforming growth factor-β (TGFβ). TGFβ is involved in cell cycle control and apoptosis [261,262]. TGFβ can interfere with the cascade of events in the GI phase described above and inhibit the ability of cells to enter S phase when the factor is present during the GI phase. TGFβ has been shown to suppress *c-myc*, cyclin A, cyclin E, and cdk2 and cdk4 expression [262]. In line with this, TGFβ has been reported to inhibit phosphorylation of $p110^{RB}$ [263]. Vitamin $D_3$ compounds induce dephosphorylation of the retinoblastoma gene product, and vitamin $D_3$ growth inhibition of MCF-7 breast cancer cells is inhibited by a TGFβ neutralizing antibody [264]. 1,25-$(OH)_2D_3$ and several analogs stimulated the expression of TGFβ mRNA and secretion of active and latent $TGF\beta_1$ by the breast cancer cell line BT-20 [154]. 1,25-$(OH)_2D_3$ enhanced $TG\beta_1$ gene expression in human keratinocytes [265] and the secretion of TGFβ in murine keratinocytes [266]. In both studies, antibodies against TGFβ inhibited the growth inhibitory effect of vitamin $D_3$. Further evidence for a vitamin $D_3$-TGFβ interaction is that bone matrix of vitamin D–deficient rats contains substantially less TGFβ than controls [267]. Therefore, on the basis of these consistent findings, TGFβ is a likely candidate to play a role in the 1,25-$(OH)_2D_3$-induced growth inhibition [268].

Interactions with the insulin-like growth factor (IGF) system have also been described. IGFs are potent growth stimulators of various cells, and their effect is regulated via a series of IGF binding proteins (IGFBPs). 1,25-$(OH)_2D_3$ and the analog EB1089 inhibit the IGF-I-stimulated growth of MCF-7 breast cancer cells [269]. In prostate cancer cell lines, 1,25-$(OH)_2D_3$ induced expression of IGFBP6 but not IGFBP4 [270]. In human osteosarcoma cell lines, 1,25-$(OH)_2D_3$ and the analog 1α-dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol potently stimulated the expression and secretion of IGFBP3 [271–273]. In one study an association has been made between increased IGFBP3 levels and 1,25-$(OH)_2D_3$ growth inhibition [271]. Recent observations that antisense oligonucleotides to IGFBP3 prevented growth inhibition of prostate cancer cells by 1,25-$(OH)_2D_3$ [235] provided further evidence for an interplay between 1,25-$(OH)_2D_3$ and IGFBP3. Interestingly, in the human osteosarcoma cell line MG-63, 1,25-$(OH)_2D_3$ and TGFβ synergistically increased IGF-BP-3 secretion [273]. An example of growth factor receptor regulation by 1,25-$(OH)_2D_3$ concerns the epidermal growth factor (EGF) receptor. This receptor is down-regulated in T47-D breast cancer cells and up-regulated in BT-20 breast cancer cells. Nevertheless, 1,25-$(OH)_2D_3$ inhibits the growth of both

cell lines [274,275]. These data provide evidence that interactions with growth factors are only part of the 1,25-$(OH)_2D_3$ action on tumor cells.

As described above, it is clear that 1,25-$(OH)_2D_3$ has effects on the expression of various oncogenes and tumor suppressor genes and that multiple interactions with various growth factors exist. However, the data on these aspects, separately as well as in combination, are still too limited to define a distinct mechanism of action for the 1,25-$(OH)_2D_3$ anticancer effects. However, with respect to growth inhibition, at this time two models of action can be postulated. In the first one, 1,25-$(OH)_2D_3$ directly interferes with a crucial gene(s) involved in the control of the cell cycle. In this case, in view of the general pattern of the genes involved in cell cycle control, this mechanism of action will be similar in all types of cancer cells. However, the effect on cell cycle genes will be dependent on the presence or absence of additional growth factors. This will determine, depending on which growth factors are present, the differences in 1,25-$(OH)_2D_3$ action between cancer types of different origin but also within cancer types of similar origin. The second model is based on an indirect effect of 1,25-$(OH)_2D_3$ on cell cycle progression and tumor growth. In this case 1,25-$(OH)_2D_3$ may either inhibit or potentiate the effect of growth stimulatory or inhibitory factors, respectively, via, for example, effects on growth factor production, growth factor binding protein levels, or receptor regulation. It is also conceivable that a combination of both models forms the basis of 1,25-$(OH)_2D_3$ regulation of tumor cell growth.

## IV. COMBINATION THERAPY

The data obtained with 1,25-$(OH)_2D_3$ and its analogs on growth inhibition and stimulation of differentiation offer promise for their use as an endocrine anticancer treatment. Single agent treatment with low calcemic 1,25-$(OH)_2D_3$ analogs could be useful; however, combination therapy with other tumor effective drugs may provide an even more beneficial effect. Up to now several *in vitro* and *in vivo* studies have focused on possible future combination therapies with 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs.

For breast cancer cells the combination of the presently most widely-used endocrine therapy, the antiestrogen tamoxifen, with 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs resulted in a greater growth inhibition of MCF-7 and ZR-75-1 cells than treatment with either compound alone [118,192,247]. In combination with tamoxifen, the cells were more sensitive to the antiproliferative action of 1,25$(OH)_2D_3$ and the analogs; that is, the $EC_{50}$ values of the vitamin $D_3$ compounds in the presence of tamoxifen were lower than those in the absence of tamoxifen. Studies with MCF-7 cells suggested a synergistic effect of 1,25$(OH)_2D_3$ and tamoxifen on apoptosis [276]. In addition, in *in vivo* breast cancer models a synergistic effect of the tamoxifen-1,25$(OH)_2D_3$ analogs combination was observed [118,119]. Additional data on the interaction between the estrogen/antiestrogen system and vitamin D comes from studies showing the presence of an estrogen responsive element in the VDR promoter and regulation of VDR by estradiol in breast cancer cells [277]. This is intriguing that the stimulator of breast cancer cell growth induces the expression of the receptor for a growth inhibitor. VDR up-regulation in breast cancer cells and increased transcriptional activity was mimicked by the phytoestrogens resveratrol and genistein and blocked by tamoxifen [278]. In colon cancer also, VDR up-regulation by estradiol has been reported. However, in colon it was hypothesized to contribute to the protective effect of estradiol on chemically-induced colon carcinogenesis [279].

These important and complex interactions between the vitamin D and estrogen endocrine system in the regulation of cancer (cells) are promising and warrant further detailed analyses, e.g. regarding tissue(cancer)-specific effects. In addition, the estrogen endocrine system may regulate the metabolism of 1,25-$(OH)_2D_3$ in cancer cells and thereby affect its action (see Section V). Interaction with another sex steroid, testosterone, has been described for ovarian cancer. Vitamin D inhibits dihydrotestosterone (DHT) and DHT stimulation of ovarian cancer cells [280]. Intriguingly, also here the growth stimulator and growth inhibitor mutually up-regulate each others receptors. Also, in prostate cancer cells, it has been shown that 1,25-$(OH)_2D_3$, while inhibiting androgen stimulated growth, up-regulates the androgen receptor [281].

Interaction with another steroid in regulating cancer cells had already been reported in 1983. The synthetic glucocorticoid, dexamethasone, and 1,25-$(OH)_2D_3$ synergistically induced differentiation of murine myeloid leukemia cells [282]. This was supported by *in vitro* and *in vivo* data showing that dexamethasone enhanced the effect of vitamin D on growth inhibition, cell cycle arrest, and apoptosis of squamous carcinoma cells [283,284]. A possible mechanism is the up-regulation of VDR by dexamethasone [283]. An interesting aspect of this combination is not only the direct interaction at cancer cell level, but also in the control of the calcemic action of 1,25-$(OH)_2D_3$. Glucocorticoids inhibit intestinal calcium absorption and increase renal calcium excretion and in this way it may limit the hypercalcemic action of 1,25-$(OH)_2D_3$ [285].

Combination of vitamin $D_3$ and retinoids has been examined in various systems. A combination of retinoic acid and 1,25-$(OH)_2D_3$ resulted in a more profound inhibition of both T47-D breast cancer cells [286] and LA-N-5 human neuroblastoma cells [287]. 9-cis-Retinoic acid augmented 1,25-$(OH)_2D_3$-induced growth inhibition and differentiation of HL-60 cells [288]. Besides growth inhibition and differentiation effects, the combination of 1,25-$(OH)_2D_3$ and various isomers of retinoic acid were more potent in reducing angiogenesis than either compound alone [146–148]. The background of the interaction between retinoids and 1,25-$(OH)_2D_3$ may be attributed to heterodimer formation of the respective receptors [289].

For several cytokines, interactions with 1,25-$(OH)_2D_3$ have been described. Interferon-γ and 1,25-$(OH)_2D_3$ synergistically inhibited the proliferation and stimulated the differentiation of HL-60, WEHI-3, and U937 myeloid leukemia cells [290–293]. Treatment of LLC-LN7 tumor cells with 1,25-$(OH)_2D_3$ with IFN-γ synergistically reduced tumor granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion and a blockage in the capacity of the tumor cells to induce granulocyte-macrophage-suppressor cells [99]. In the mouse myeloid leukemia cell line MI interleukin-4 enhanced 1,25$(OH)_2$ $D_3$-induced differentiation [189,294,295]. Also with interleukin-1β, interleukin-3, interleukin-6, and interleukin-12 interactions with 1,25-$(OH)_2D_3$ have been reported [296–298]. 1,25-$(OH)_2D_3$ and tumor necrosis factor synergistically induced growth inhibition and differentiation of HL-60 [299]. For MCF-7 cells an interaction between 1,25-$(OH)_2D_3$ and tumor necrosis factor has also been reported [298,300]. In the presence of GM-CSF, lower concentrations of 1,25$(OH)_2D_3$ could be used to achieve a similar antiproliferative effect in MCF-7 cells [301] and to induce differentiation of U937 myeloid leukemic cells [302]. Other factors shown to interact with 1,25-$(OH)_2D_3$ are butyrate [303–305], melatonin [306], EGF [307], and the factors described in Section III.C.

Furthermore, combinations of vitamin $D_3$ compounds with cytotoxic drugs, antioxidants, and radiation have been studied. *In vivo* adriamycin and *in vitro* carboplatin, cisplatin, and doxorubicin interacted synergistically with 1,25-$(OH)_2D_3$ to inhibit breast cancer cell growth [113,308–311]. In a carcinogen-induced rat mammary tumor model, treatment with 1α-$(OH)D_3$ and 5-fluorouracil, however, did not result in enhanced antitumor effects [96]. Recently, interactions with a plant-derived polyphenolic antioxidant, carnosic acid were demonstrated in the differentiation of HL-60 cells, which was related to a decrease in the intracellular levels of reactive oxygen species [312,313]. Also interaction with radiation therapy in breast cancer has been described [314–316].

The data on combinations of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs with various other anticancer compounds are promising and merit further analyses. The development of effective combination therapies may result in better response rates and lower required dosages, thereby reducing the risk of negative side effects.

## V. RESISTANCE AND VITAMIN D METABOLISM

Classic vitamin D resistance concerns the disease hereditary vitamin D–resistant rickets, which is characterized by the presence of a nonfunctional VDR and consequently aberrations in calcium and bone metabolism (see Chapter 72). For cancer cells, the presence of a functional VDR is also a prerequisite for a growth regulatory response, and a relationship between VDR level and growth inhibition has been suggested for osteosarcoma, colon carcinoma, breast cancer, prostate cancer cells, and rat glioma [1,2,108,129,205,210,317–321]. Cell lines established from DMBA-induced breast tumors in VDR knockout mice are insensitive to growth arrest and apoptosis by 1,25-$(OH)_2D_3$, EB1089 and CB1093 [322]. Albeit that VDR is a prerequisite for tumor cell growth regulation, the presence of the VDR is not always coupled to a growth inhibitory response of 1,25$(OH)_2D_3$. Results from studies with transformed fibroblasts [194], myelogenous leukemia cells [190,204,323], transformed keratinocytes [187], and various breast cancer cell lines [211,324] demonstrated a lack of growth inhibition by 1,25$(OH)_2D_3$ even in the presence of VDR. In this situation, the designation "resistant" is based on the lack of growth inhibition, even though, as discussed earlier in Section III.C, some of these cells are still capable of being induced to differentiate [204,211]. This points to a specific defect in the growth inhibitory pathway. In the resistant MCF-7 cells, this defect is not located at a very common site in the growth inhibitory pathway of the cell because the growth could still be inhibited with the antiestrogen tamoxifen [324]. For myelogenous leukemia cells, similar observations have been made [325].

For VDR-independent resistance to growth inhibition, the underlying mechanism(s) is unknown. For the resistant MCF-7 clone, this is not related to up-regulation of the P-glycoprotein [324]. Interestingly, these vitamin D–resistant MCF-7 clones can be sensitized to 1,25$(OH)_2D$ by activation of protein kinase C, resulting in induction of apoptosis and transcriptional activation, suggesting that alterations in phosphorylation may affect vitamin D sensitivity [326]. An interesting growth

inhibition resistant MCF-7 cell clone was described by Hansen *et al.* This clone was not growth inhibited while VDR was still present and 24-hydroxylase could still be induced [327]. Other examples of vitamin D resistance are HL60 cells that have been cultured for four years in the presence of 1,25-$(OH)_2D_3$ and resulted in clones that are resistant to differentiation inducing and growth inhibition. They became not only resistant to 1,25$(OH)_2$D but also to 5-beta-D-arabinocytosine, suggesting a common metabolic pathway being responsible [328]. Whether this relates to the up-regulation of the multidrug resistance proteins is not clear. In the resistant leukemia $JMRD_3$ cell line, altered regulation and DNA–binding activity of *junD* as part of the AP-1 complex has been reported [200]. Resistance to growth inhibition in the presence of VDR has also been linked to disruption of the VDR-RXR complex [329] and increased RXR degradation [330]. In addition, other factors, like the acute myeloid leukemia translocation products (e.g. PLZF) may contribute to resistance to vitamin D by sequestering the VDR [223,224].

The 1,25$(OH)_2D_3$ sensitive and resistant cell clones provide interesting models to examine the molecular mechanisms of 1,25$(OH)_2D_3$-induced growth inhibition. For example, lack of p21 results in no cell cycle block [331] and no apoptosis was detected with a mutated p53 [211]. Finally, the recent identification of cellular proteins that are involved in the vitamin D resistance in new world primates might add to the understanding of tumor cell resistance to vitamin D [332,333] (see Chapter 21).

At this time, the major mechanism for vitamin D resistance or reduced sensitivity in VDR containing tumor and cancer cells is 1,25-$(OH)_2D_3$ catabolism via the C24-hydroxylation pathway. An inverse relationship between cellular metabolism of 1,25-$(OH)_2D_3$ via 24-hydroxylation and growth inhibition of prostate cancer cells has been suggested [318]. The latter observation is intriguing, the more so as an inverse relationship between VDR level and induction of 24-hydroxylase (CYP24) activity was reported. In general, there may exist a direct relationship between VDR level and induction of 24-hydroxylase activity [319,334]. An important role in the control of 1,25-$(OH)_2D_3$ action on cancer cells was provided by studies with the 1,25-$(OH)_2D_3$–resistant prostate cancer cell line DU145. It was shown that 1,25-$(OH)_2D_3$ did inhibit the growth of these cells when it was combined with the 24-hydroxylase inhibitor Liazorole [335]. Inhibition of 24-hydroxylase activity in HL-60 cells also altered the effect of 1,25-$(OH)_2D_3$ and 20-epi analogs [336]. The action of the analog EB1089 was also limited by hydroxylation at the C24 position [337]. However, it was suggested that the increased potency of EB1089 is at least partly due to resistance to 24-hydroxylation [234]. Alternatively, 24-hydroxylation of the analog KH1060 has been implicated as one of the mechanisms to explain the potency of this analog. The 24-hydroxylated metabolites of this analog are very stable and are biologically active [338,339]. It has been shown that the naturally occurring 24-hydroxylated metabolite of vitamin $D_3$, 24R,25-$(OH)_2D_3$, also has a preventive effect on chemically-induced colon cancer [340].

Interaction between the estrogen system and 24-hydroxylase is also of importance. Recent data have shown that the phytoestrogen genistein inhibits 24-hydroxylase activity in prostate cancer cells and thereby increases the responsiveness to 1,25-$(OH)_2D_3$ [341]. A role for 24-hydroxylase as oncogene is suggested by data showing amplification of the CYP24 locus on chromosome 20q13.2 [342].

In contrast to degradation of 1,25-$(OH)_2D_3$ by 24-hydroxylase in cancer cells, recently it has become clear that tumor cells contain 1α-hydroxylase activity and thereby are able to generate 1,25-$(OH)_2D_3$. Expression of 1α-hydroxylase has been demonstrated in colorectal cancer [343–345]. It was postulated that in early stages tumor cells respond by up-regulating 1α-hydroxylase activity to counteract neoplastic growth while at later stages of tumor development this is lost [343]. Also in prostate cancer [346] and inflammatory myofibroblastic tumor [347] 1α-hydroxylase has been detected, albeit in the latter case the tumor contains large numbers of macrophages. It can be anticipated that in the coming years investigation of the expression of both 24-hydroxylase, 1α-hydroxylase in tumors will add to the understanding of vitamin D in the initiation and progression of cancer.

## VI. STIMULATION OF PROLIFERATION

Over the years a limited number of studies have demonstrated that, in contrast to growth inhibition, 1,25-$(OH)_2D_3$ can also stimulate tumor cell growth and tumor development. In several cells 1,25-$(OH)_2D_3$ has been reported to have a biphasic effect, that is, at lower concentrations ($<10^{-9}$ M) it stimulates proliferation and at higher concentrations ($10^{-9}$ to $10^{-7}$ M) it inhibits proliferation. However, clear growth stimulation can sometimes be observed not only at low concentrations but also at the concentrations generally found to inhibit tumor cell proliferation and tumor development. 1,25-$(OH)_2D_3$ has been shown to stimulate the growth of a human medullary thyroid carcinoma cell line [348]. Not only cancer cells but also several normal cells, for example, human monocytes [349], smooth muscle

cells [350], and alveolar type II cells [351], are stimulated to grow by 1,25-$(OH)_2D_3$.

Skin is another organ in which different effects of 1,25-$(OH)_2D_3$ have been observed. *In vivo* studies demonstrated that 1,25-$(OH)_2D_3$ and analogs stimulate keratinocyte proliferation in normal mice [352–355] and enhance anchorage-independent growth of preneoplastic epidermal cells [356]. In contrast, other studies showed 1,25-$(OH)_2D_3$ inhibition of proliferation of mouse and human keratinocytes [357,358], and 1,25-$(OH)_2D_3$ is also effective in the treatment of the hyperproliferative disorder psoriasis [359]. Moreover, *in vivo* studies demonstrated that, depending on the carcinogen, 1,25-$(OH)_2D_3$ can either reduce [88] or enhance the induction and development of skin tumors in mice [360,361]. In addition, 1,25-$(OH)_2D_3$ enhances the chemically-induced transformation of BALB 3T3 cells and hamster embryo cells [362,363]. 1,25-$(OH)_2D_3$ also enhanced 12-O-tetradecanoylphorbol-13-acetate-induced tumorigenic transformation of mouse epidermal JB6 Cl41.5a cells [364,365].

Another example comes from research on osteosarcoma cells. In 1986 it was shown that 1,25-$(OH)_2D_3$ stimulated the growth of tumors in athymic mice inoculated with the ROS 17/2.8 osteosarcoma cell line [366]. Earlier the same group reported growth stimulation *in vitro* of these osteosarcoma cells at low concentrations of 1,25$(OH)_2D_3$, but growth inhibition by $10^{-8}$ M [317]. They speculated that this discrepancy resulted from limited *in vivo* availability of 1,25-$(OH)_2D_3$ for the tumor cells, resulting in concentrations shown to be growth stimulatory *in vitro*. However, in other experiments with nude mice, the availability of 1,25-$(OH)_2D_3$ did not seem to be a factor, as growth inhibition was observed (see Table II). In particular, in nude mice implanted with human osteosarcoma cells (MG-63), growth inhibition and tumor suppression by 1,25-$(OH)_2D_3$ were observed [98]. In two different *in vitro* studies, growth inhibition of MG-63 and growth stimulation of ROS 17/2.8 cells was reported [367,368]. For smooth muscle cells, it has been demonstrated, for example, that growth inhibition or stimulation can depend on the presence of additional growth factors in the culture medium [350]. We followed up on this concept by comparing the effects of 1,25-$(OH)_2D_3$ and analogs on the growth and osteoblastic characteristics of the two osteosarcoma cell lines under identical culture conditions. At concentrations $10^{-10}$ to $10^{-7}$ *M*, 1,25-$(OH)_2D_3$ caused an increase in cell proliferation by 100% in ROS 17/2.8 cells, whereas the proliferation of MG-63 cells was inhibited (Fig. 2) [197]. In contrast, in both cell lines 1,25$(OH)_2D_3$ stimulated osteoblastic differentiation characteristics such as production of osteocalcin and alkaline phosphatase activity [197,367]. Analyses with another steroid hormone demonstrated that glucocorticoids inhibited the growth of both osteosarcoma cell lines [369,370]. These data indicate specific differences between these cell lines, especially with respect to the 1,25-$(OH)_2D_3$ growth regulatory mechanisms.

Taken together, the data on growth stimulation and tumor development, although detected in only a minority of cancer cells, demonstrate that treatment with 1,25-$(OH)_2D_3$ or analogs may not always cause growth inhibition and tumor size reduction. It is therefore of utmost importance to identify the mechanism(s) by which 1,25-$(OH)_2D_3$ exerts its inhibitory and stimulatory effects on cell growth. This may provide tools to assess whether treatment of a particular tumor will be beneficial. Moreover, purely from a mechanistic point of view, the presence of growth-stimulated and growth-inhibited cells, like the 1,25-$(OH)_2D_3$ sensitive and resistant cells, may provide tools to examine the 1,25-$(OH)_2D_3$ mechanism of growth regulation.

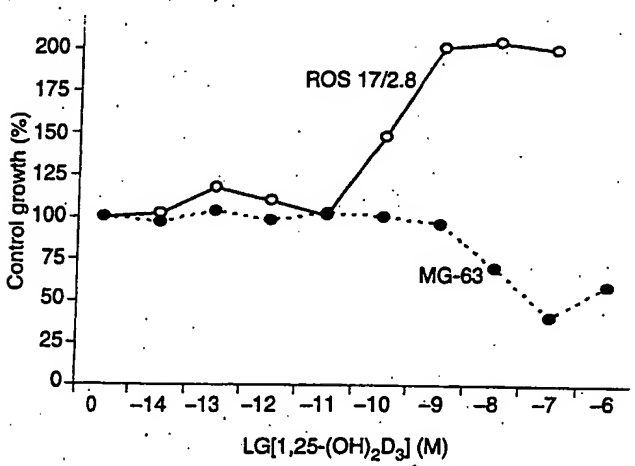



FIGURE 2 Effect of 1,25-$(OH)_2D_3$ on proliferation of the osteosarcoma cell lines ROS 17/2.8 and MG-63. Effects on proliferation were examined as described by van den Bemd *et al.* [197].

## VII. CONCLUSIONS

The data obtained so far, on (1) the distribution of the VDR in a broad range of tumors and (2) the inhibition of cancer cell growth, angiogenesis, metastasis, and PTHrP synthesis by 1,25-$(OH)_2D_3$, all hold promise for the development of treatment strategies based on vitamin $D_3$ use in a wide range of cancers. Moreover, combination of vitamin D compounds with other antitumor drugs, hormones, or growth factors is an important additional therapeutic option. Throughout the last years data have accumulated on the cellular targets and mechanism of action of 1,25-$(OH)_2D_3$–induced cancer

growth inhibition. The clinical application is enhanced by the development of 1,25-$(OH)_2D_3$ analogs with potent growth inhibitory actions and reduced hypercalcemic activity. At the moment more clinical studies are needed in order to firmly establish whether 1,25$(OH)_2D_3$ and especially vitamin $D_3$ analogs have therapeutic potential. In the meantime it is crucial to further our understanding of the mechanism(s) by which 1,25$(OH)_2D_3$ exerts its effects on tumor cell growth so that these drugs may be employed more effectively.

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